#11

PATENT
Attorney Docket No.: 018941-000710US
Client Ref. No.: B99-017-2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Peggy Lemaux, David McElroy and Thomas Koprek

Application No.: 09/384,811

Filed: August 27, 1999

For: TRANSPOSON TAGGING AND GENE DELIVERY IN SMALL GRAIN CEREALS

Examiner: Cynthia Collins

Art Unit: 1638

SUPPLEMENTAL SUBMISSION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Further to Applicants' response mailed December 3, 2002, enclosed is a signed copy of the Declaration by Peggy Lemaux, Ph.D., submitted pursuant to 37 C.F.R. § 1.132.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

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#14 12/13/02

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DECLARATION UNDER 37 C.F.R. §1.132 BY PEGGY LEMAUX, Ph.D.

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

1. I, Peggy Lemaux, Ph.D., am a Cooperative Extension Specialist at the University of California, Berkeley and am currently the Director of the University of California Division of Agriculture and Natural Resources Biotechnology Workgroup. I am a co-inventor of the subject matter of the above-referenced patent application. I have worked in the field of plant genetics for over 15 years.

2. I hold a Ph.D. from the University of Michigan, which was conferred in 1977. I have authored over 65 publications relating to microbial and plant genetics and molecular biology. A copy of my curriculum vitae is attached as Exhibit 1.

3. I have read and am familiar with the contents of the above-referenced patent application and claimed subject matter. It is my understanding that the Examiner has rejected the claims as allegedly unpatentable over the prior art. In particular, the Examiner believes that the invention is obvious over the combination of various prior art publications, of which the primary references are McElroy *et al.* "Development of a simple transient assay for *Ac/Ds* activity in cells of intact barley tissue" *The Plant Journal* 11:157-165, 1997), Wan *et al.* ("Generation of Large Numbers of Independently Transformed Fertile Barley Plants" *Plant Physiol.* 104:37-48, 1994), and Bancroft *et al.* ("Development of an efficient two-element transposon tagging system in *Arabidopsis thaliana*" *Mol. Gen. Genet.* 233:449-461, 1992). The Examiner alleges that the success of Wan *et al.* in transforming barley, the success of McElroy *et al.* in demonstrating Ac transposase-mediated excision of *Ds* in barley cells, and the use of the *Ac/Ds* transposase for stable transformations in a number of other systems, *e.g., Arabidopsis*, would lead one in the art to use the system in barley with a reasonable expectation that it would work in barley.

4. This declaration is submitted to provide further evidence that prior to Applicants' invention, one of skill could not reasonably expect the *Ac/Ds* system to generate stable transformants in barley, *i.e.,* transformants in which the transposable element can be reactivated and reinsert into the genome, because of certain characteristics of the barley cell and genome, *e.g.*, the amount of methylation and gene silencing.

5. The claims under prosecution are drawn to plants containing an *Ac* or *Ds* transposon stably integrated into the genome and methods of transforming plants using the *Ac/Ds* system. The transposons in the claimed barley plants can be excised and reintegrate into the genome in the presence of a transposase enzyme. Although stable integration in barley plants has been obtained using other transformation systems, such as that taught by Wan *et al.*, it has been very difficult for those in the art to generate barley

transformation systems based on the *Ac/Ds* system, even though it has been used in many other plants. In order for the *Ac/Ds* system to provide usable stable transformants in barley, the elements must be introduced into plants and stably integrate into the genome. That is, they must not be subject to rearrangement, deletions, etc. over time. Further, they must retain their ability to transpose *i.e.,* the transposase must not be silenced and the recognition sites not be methylated or changed in sequence; and the *Ds* elements must retain the ability to be re-insert into the genome.

6. Prior to our invention, it was unknown whether such conditions could be met in barley. Instability of gene expression and gene silencing are believed to be due, at least in part, to methylation. The barley genome is known to be highly methylated. It was not known whether the architecture of the highly methylated genome would permit high levels of re-insertion of transposons. Further, as explained below, frequent methylation of foreign sequences has been demonstrated in barley and has been shown to frequently lead to instability and/or gene silencing. Accordingly, one could not predict whether the methylation status of barley would provide for stable, active *Ac/Ds* integration and re-integration.

Instability of foreign sequences in barley

7. McElroy *et al.* teach a transient, transgene introduction system that shows that a *Ds* transposable element can be excised from a reporter plasmid in barley cells in the presence of *Ac* transposase activity from an Ac transposase gene introduced via bombardment at the same time. However, transposable elements can undergo deletions, internal rearrangements and/or methylation-mediated inactivation converting an active, movable element into an element incapable of movement. Critically, the reference is silent on whether the excised *Ds* elements could re-integrate into the barley genome, a feature vital for effective transposon tagging or gene delivery.

8. It has long been known that barley is highly methylated and that methylation plays a role in instability of foreign sequences in barley. In an exemplary study, Rogers & Rogers (*Plant Mol. Biol.* 18:945-961, 1992, submitted herewith as Exhibit 2) tested the effect of methylation of foreign DNA on its stability after introduction into barley plants. The investigators noted that foreign DNA could be introduced into barley cells. Further the foreign DNA persisted through at least two plant generations. However, they pointed out that this persistence was not equivalent to stable transformation and that the DNA was frequently rearranged or lost in subsequent generations. They attributed the instability, at least in part, to methylation. They further noted that cereals have an unusual genome organization where structural gene sequences are very GC-rich. Methylation systems that are endogenous to barley are in part dependent on the GC-rich nature of the genome. Foreign sequences, however, do not share this feature. They demonstrated that foreign sequences were methylated in a specific pattern that was distinguishable from the methylation pattern of the highly GC-rich barley sequences and concluded that the foreign sequences could therefore be easily distinguished from endogenous sequences and thus preferentially inactivated.

Loss of activity in barley

9. Inactivation of foreign sequences by methylation may lead not only to direct instability and loss of a sequence from the genome, but may also lead to lack of activity of the encoded protein or compromise the ability of the introduced sequence to perform its function. Gene silencing is thought by those in the art to be due to methylation of regulatory sequences or, in some cases, the coding sequences of genes. Thus, methylation may silence expression of a gene, for example, an *Ac* transposase gene or a transposition cassette associated with a *Ds* element. Further, methylation can lead to direct inactivation of transposition, for example, by preventing a *Ds* element from stably integrating and retaining the ability to reinsert into another region of the genome in the presence of transposase activity. Lastly, it is also believed that the inverted repeats in the

Ds elements, needed for recognition by *Ac* transposase and transposition, can trigger methylation-induced silencing that prevents re-activation of *Ds*. Thus, methylation in barley could also result in gene silencing.

10. Not only is the ability of the *Ds* element to re-insert important for transposon tagging and expression, it is also important in the generation of plants in which it is desirable to lose the transformation vector or selectable marker sequences. The latter capability can be used to obtain integration of a transgene contained within *Ds* inverted repeat ends at a position unlinked to the site at which the transformation vector originally integrated. This means that in subsequent generations, plants may be obtained that contain only the *Ds* inverted repeat ends and the inserted transgene of interest and not the other nucleic acid sequences contained in the original transformation vector. Retaining the activity of the transgene of interest and its ability to relocate under the control of *Ac* transposase requires that expression persist through multiple generations.

Conclusion

11. Thus, even though McElroy *et al.* showed in a transient assay system that *Ac* transposase is active in barley and can excise a *Ds* cassette bearing the inverted repeats and a transgene of interest from plasmids that are transiently introduced into barley, there is no teaching or suggestion that *Ds* can excise and then re-integrate in subsequent generations in the presence of transposase activity. Further, neither Wan *et al.* nor Bancroft *et al.* provide any teaching or suggestion that the *Ds* element will be able to reintegrate in the highly methylated barley genome or will not itself become methylated and incapable of excising or reintegrating in a stably transformed barley cell.

12. Therefore, for the reasons provided above, one of skill in the art, at the time the application was filed, would not have been able to use the *Ac/Ds* transposon system in barley with a reasonable expectation of success for obtaining barley plants containing an

Ac or *Ds* transposon stably integrated into the genome where the Ds element can excise and reintegrate into the genome in the presence of transposase.

13. I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that +these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon.

Date: _____ By: _____

Peggy Lemaux, Ph.D.

CURRICULUM VITAE

Peggy Goodenow Lemaux

Education:

Miami University, Oxford, Ohio: B.A., cum laude (microbiology, chemistry) 1964-1968.
University of Michigan, Ann Arbor, Michigan: M.S. (microbiology) 1968-1969.
University of Michigan, Ann Arbor, Michigan: Ph.D. (microbiology) 1972-1977.
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York: Molecular Biology of Plants Course 1983.

Employment:

Research Assistant: Infectious Diseases Unit, The Upjohn Company, Kalamazoo, Michigan 1969-1972.
Staff Associate: Department of Plant Biology, The Carnegie Institution of Washington, Stanford, California. 1985-1987.
Research Scientist: Department of Plant Genetics, DeKalb/Pfizer Genetics, Groton, Connecticut 1987-1988.
Senior Research Scientist: Department of Plant Genetics, DeKalb Plant Genetics, Groton, Connecticut 1988-1991.
Associate Cooperative Extension Specialist: University of California, Berkeley, California 1991-1996.
Associate Director, University of California Systemwide Biotechnology Research and Education Program, University of California Office of the President 1994-1995.
Cooperative Extension Specialist: University of California, Berkeley, California 1996-present.
Director, University of California Division of Agriculture and Natural Resources Biotechnology Workgroup, 1999-present

Teaching/Outreach/Campus/Professional Activities:

Member, Legislative and Regulatory Subcommittee of UC Systemwide Biotechnology Research and Education Program 1992-present.
Member, Advisory Committee, Berkeley Biotechnology Education, Inc. 1992-present.
Member, Chancellor's Advisory Committee on Laboratory and Environmental Biosafety, 1992-1995.
Member, Biological Sciences Advisory Committee, National Aeronautics and Space Administration, 1993-1997.
Member, Curriculum Development Committee, Berkeley Unified School District/Vista Community College Two-Plus-Two Technical Curriculum for Biotechnology, 1993-1995.
Member, Program Planning Advisory Committee, Division of Agriculture and Natural Resources, University of California, 1994-1997.

Member, Standing Committee, College of Natural Resource, 1994-1997.
Member, National Sustainable Agriculture Advisory Committee, United States Department of Agriculture, 1995-1996.
Member, Agricultural Subcommittee of UC Systemwide Biotechnology Research and Education Program 1995-present.
Member, Division of Agriculture and Natural Resources Organizational Strategy Team, 1996-1997.
Member, Review Committee for the Crop Resources Germplasm Program, Division of Agriculture and Natural Resources, 1997.
Chair, member, Public Affairs Committee, American Society of Plant Biologists, 1997-present
Chair, elected member, American Association for the Advancement of Science, Electorate Nominating Committee, 1999-
Member, American Society of Microbiology, Public and Scientific Affairs Board Committee on Food and Agriculture. 1999-present
Chair, UC Division of Agriculture and Natural Resources, Statewide Workgroup, Linking Research and Education in Agricultural and Environmental Biotechnology, 1999-
Co-Chair, UC Division of Agriculture and Natural Resources, Statewide Workgroup, Nutritional Genomics, 1999-
Member, California Food Biotechnology Advisory Committee, 2001-

Outreach Publications:

Odegard, W., Townsend, M. S., Lemaux, P. G., Disbrow, D. and Chang, G. 1994. "Biotechnology and Food", 4-H What's In Food Family Food Safety Education Program.

Huttner, S., H.I. Miller, P.G. Lemaux 1995 "U.S. Agricultural Biotechnology: Status and Prospects", *Technology Forecasting and Social Change*, 50:25-39.

Lemaux, P.G., M.J. Cho, J. Louwerse, R. Williams, Y. Wan 1996 "Bombardment-mediated Transformation Technologies for Barley", *Bio-Rad Laboratories Technical Bulletin, Bulletin* #2007.

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Lemaux, P.G. 1996. "Biotechnology in Cotton - Current Status". *California Cotton Review* 41:1-2.

Sellers, C. and P.G. Lemaux 1996 "Development and Use of Herbicide Resistant Crops for California" *Proceedings of the 48th annual California Weed Science Society*, Sacramento CA. pp. 212-223.

Vickers, K.M. and P.G. Lemaux 1996 "Impact of Biotechnology and the Environment: Challenges and Opportunities". *Proceedings from the American Society for Horticultural Science 93rd Annual Conference. HortScience* 31:698-699. 1996.

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Lemaux, P.G. 1997 "Transformation of Recalcitrant Crops: Progress and Remaining Challenges", *Proceedings of American Society of Sugarbeet Technologists*, March 1997.

Lemaux, P.G. 1999 "Impact of Public Perception on Regulatory Policy for Agricultural Biotechnology", *Plant Biotechnology*, 16:73-78.

Lemaux, P.G. and Qualset, C.A. 1999. Wheat of the Past and Wheats of the Future. *Aaronsohn Lectures on Wild Emmer Wheat*, Tel Aviv University Institute for Cereal Crops Improvement, April 14-15, 1999, Zikhron Ya'aqov, Israel. pp. 125-139.

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Cho, M.-J. and Lemaux, P.G. 2000. Issues with new GM foods: biotechnology and agriculture. *Biotechnology and Seed War in the 21st Cenutry*, Seoul, Korea, pp. 65-75.

Lemaux, P.G. 2002. Ignorance about bio-tech hurts world's hungry. *Duluth News-Tribune*, August 1, 2002. http://www.duluthsuperior.com/mld/duluthsuperior/3777424.htm

Professional Societies:

American Association for the Advancement of Science
American Society of Agronomy
American Society of Microbiology
American Society of Plant Physiologists
Council for Agricultural Science and Technology
Crop Science Society of America
International Society of Plant Molecular Biologists
National Science Teachers Association
Soil Science Society of America

Honors

Honored Women of the University of California, Berkeley. April 29, 1995

Distinguished Service Award, Outstanding Research, from the Cooperative Extension Academic Assembly Council, Division of Agriculture and Natural Resources, Outstanding Research, DANR August 1997

Fellow, American Association for the Advancement of Science

Patents

Cho, Myeong-Je; del Val, Gregorio; Caillau, Maxime; Lemaux, Peggy G.; Buchanan, Bob B. Barley gene for Thioredoxin and NADP-thioredoxin reductase. Official Gazette of the United States Patent and Trademark Office Patents. Apr. 30, 2002. 1257(5):No Pagination. US 6380372; April 30, 2002, 536-231, USA The Regents of the University of California.

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The importance of DNA methylation for stability of foreign DNA in barley

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Abstract

We developed a system using 'passive' reporter genes driven by aleurone tissue-specific promoters to test the effect of methylation of foreign DNA on its stability after introduction into barley plants. The foreign DNA was introduced into barley cells and was able to persist through at least two plant generations. Transformation of barley cells was defined by showing initiation of transcription at the proper site on the barley promoter for the chimeric gene in aleurone tissue from both a primary transformant and its progeny, and by tissue-specific expression (aleurone > leaf) in the progeny. This persistence through many multiples of cell division is formally equivalent to transformation, regardless of whether the DNA was chromosomally integrated or carried as an episome, but did not necessarily represent stable integration into the genome since the foreign DNA was frequently rearranged or lost. The foreign DNA was most stable when plasmid DNA used in transformation lacked dA methylation but had complete methylation of dC residues in the CG dinucleotide at *Hpa* II sites; dA methylation alone was associated with marked foreign DNA instability. Our results are consistent with the presence of a previously undefined system in barley that identifies DNA lacking the proper methylation pattern and causes its loss from actively dividing cells. These results may be important when applied to different strategies using selectable markers for cereal transformation.

Introduction

Stable transformation of monocotyledonous plants, notably the agriculturally important cereals, has been difficult to achieve (reviewed in [30]). This has been puzzling because a number of different reports indicate that it may not be difficult to transfer foreign DNA into cereal plants where it is, at least transiently, expressed. For example, Grimsley *et al.* [14] demonstrated that *Agrobacterium* can readily transfer the DNA for maize streak virus into maize seedlings, where it is transcribed to produce infectious virus; similar results were obtained with other cereals [6]. Other workers have assayed the production of a specialized class of amino acids, opines, that are synthesized in plant cells by genes transferred from the *Agrobacterium* Ti plasmid, and concluded that *Agrobacterium*-mediated DNA transfer to maize cells occurred when those bacteria were inoculated into

wounded tissue on seedlings [13]. In spite of these observations, only cultured rice tissue has been permanently transformed by *Agrobacterium*-mediated DNA transfer [31]. The apparent contradiction between ready transfer of DNA but poor results with permanent transformation has not been explained, although theories have been proposed [30].

Other approaches have suggested that it may be relatively easy to achieve transfer of foreign DNA into cells in mature [29] or developing [9] floral parts of maize and rye respectively. In the former reference, DNA from a donor maize line with one kernel phenotype was mixed with pollen from a second, recipient maize line and applied to the recipient plant's silks. Uptake and expression of the donor DNA was assessed by visually scoring the phenotype of the resulting kernels; it was concluded that as many as 9% of the resulting kernels expressed the exogenous DNA [29]. This interpretation has been criticized because no molecular techniques were used to corroborate the results, and attempts to use markers on exogenous plasmid DNA in similar approaches have been unsuccessful [30]. In the second example, plasmid DNA carrying a bacterial gene for kanamycin resistance under control of the nopaline synthase promoter was injected into developing tillers of rye plants in an attempt to get uptake of the DNA into cells that later would participate in pollination and formation of transformed grains [9]. From ca. 3000 resulting grains, three plants were obtained that grew in the presence of kanamycin, expressed the aminoglycoside phosphotransferase enzyme activity in leaf tissue, and had appropriately hybridizing restriction fragments in genomic DNA as assessed on Southern blots [9]. The authors' conclusion that these plants were transformed was questioned because no transmission to progeny was demonstrated, and because others have not been able to reproduce the results with other cereals [30].

Our results, presented below, provide an explanation on the molecular level why both DNA transferred from *Agrobacterium* and other plasmid DNA might be unstable in cereal cells. These experiments grew out of our attempt to reproduce the results of de la Peña *et al.* [9] in barley. We constructed 'passive' reporter genes for transformation experiments, where the promoters were taken from two barley α-amylase genes [18, 34] that are expressed at high levels in the aleurone layer of the grain endosperm. These promoters were placed in front of two different coding sequences: one, that for the sweet-tasting plant protein thaumatin [11], and the other, that for *Escherichia coli* β-glucuronidase (GUS) [17]. Our strategy was to inject plasmid DNA carrying one of these marker genes into a developing tiller of a barley plant, then harvest the grain that was produced from the tiller and screen bits of aleurone tissue from each seed for expression of the appropriate marker. If a putative positive was identified, the embryo from the same grain was germinated and Southern blot analysis of DNA from the resulting plant was performed. A positive hybridization result was taken to indicate a probable transformant; we tested a total of 2400 seeds and obtained 22 positives by that criterion (S.W. Rogers and J.C. Rogers, unpublished data). We found, however, that the GUS construct DNA was rapidly lost from the growing plant, while the thaumatin marker DNA was more likely to persist in new growth on the plant and to be transmitted to the second generation.

These confusing results led to the work described below. Here we demonstrate that the foreign DNA is in barley and not in a contaminating microorganism, as judged by proper initiation on the foreign gene promoter and by preferential expression of the foreign gene in aleurone as compared to leaf tissue. We prospectively study and identify specific structural features of the transforming DNA that greatly affect stability within the plant. Our experiments show that the presence of N-6 methyladenine on the transforming plasmid DNA was associated with much more rapid loss of the DNA from new growth on the plant, while the absence of N-6-methyladenine coupled with methylation of C residues at CG dinucleotides greatly increased the stability of the same plasmid, and allowed transmission into the second generation, with persistence of the DNA in those plants.

These results, coupled with recent data that maize genomic DNA carrying three copies of an integrated kanamycin resistance gene is highly efficient in transforming maize suspension culture protoplasts [3], suggest that cereal cells have the ability to discriminate between foreign and genomic DNA, and act to delete the foreign DNA. The mechanism through which this recognition/deletion system works must involve, at least in part, analysis of methylation patterns on the foreign DNA, since our results demonstrate that the state of methylation of the transforming DNA is a major determinant of its stability within the plant cells.

Materials and methods

General methods

Methods for construction, cloning, and analysis of recombinant DNA products have been described previously [18, 38]. Southern blots were prepared by transfer onto Biotrace RP membrane (Gelman, Ann Arbor, MI) according to the manufacturer's directions and hybridized as before [18]. Preparation of total cellular RNA from aleurone and leaf tissues, and primer extension with reverse transcriptase were performed as described [32]. Amplification of specific cDNAs from the primer extension reaction was performed using the polymerase chain reaction (PCR) [12].

Barley miniprep DNA purification

For analysis of plants grown from seeds selected as possible positives in one of the screens, the second tiller from each plant was removed when it was judged to be 1–2 g in size and weighed. Miniprep DNA was prepared according to Dellaporta *et al.* [10] and resuspended for use in 1 mM EDTA pH 7.4 where the final volume of buffer was adjusted to 100 µl for each gram of tissue used. We assumed that the final concentration of DNA was approximately 1 mg/ml. Control DNAs were prepared from untransformed plants grown alongside the test plants. Restriction digests routinely used 10 µl of a DNA sample per incubation.

Recombinant marker genes

The fidelity of all coding sequence fusions was confirmed by sequencing. The final construct used in these experiments, JR214, was combined from the following two individual constructs.

JR083. The 1.6 kb promoter/upstream sequence from the low-pI α-amylase gene, Amy32b [38], extends from the 5′ *Hin*d III site to a *Bam* HI linker introduced into the *Bss* HII site immediately preceding the ATG translation start codon; this promoter cassette is identified as JR020.

JR073. The *Hin*c II-*Hin*d III fragment from plasmid pUR528 [11], encoding prothaumatin minus codons for the first 7 N-terminal amino acids was ligated into the corresponding *Hin*c II-*Hin*d III gap in the PAPI cDNA [23]. This construct, JR073, carries the 5′ untranslated sequence (with a unique *Bcl* I site immediately preceding the ATG start codon) and coding sequence for the signal peptide and first 7 amino acids of the mature form of PAPI fused to the sequence for prothaumatin beginning with the codon for residue 8. The 3′ untranslated/poly(A) addition sequences from Amy32b, as an *Nsi* I (blunt-ended with T4 DNA polymerase)-*Hin*d III fragment was ligated into the blunt-ended *Fok* I-*Hin*d III interval of JR073; the *Hin*d III site was converted to a *Sac* I site with a linker. This intermediate, on a *Bam* HI-*Sac* I fragment, was inserted into the pUC18 polylinker and transformed into a *dam*$^-$ strain of *E. coli.* The Amy32b upstream/promoter sequence, as a *Hin*d III-*Bam* HI fragment from JR020, was then inserted into the *Hin*d III-*Bcl* I interval to complete *JR083* in plasmid pUC18.

JR133. The ca. 2.0 kb 5′ fragment from the high-pI α-amylase gene, Amy6-4 [18], extending from a *Hin*d III site through the upstream/promoter and signal peptide coding sequences to a blunt-ended *Eco* NI site was ligated to the poly-

linker *Sma* I site of pBI121 [17]. This provided an in-frame fusion between the 28 N-terminal amino acids from Amy6-4 and GUS. The 3′ untranslated/poly(A) addition sequences from Amy6-4 on a ca. 200 bp *Sac* I-*Hin*d III fragment were ligated to the 3′ *Sac* I site of the GUS sequence, and the completed construct, on a ca. 4.1 kb *Hin*d III fragment, was carried in the polylinker of the 3.2 kb plasmid, Bluescribe(+) (Stratagene, San Diego, CA).

JR214. A partial *Hin*d III digest of JR133 was blunt-ended with Klenow fragment and religated. A resultant clone was identified where the 5′ *Hin*d III site had thereby been converted to an *Nhe* I site; this intermediate had an intact polylinker at the 3′ end. The *Hin*d III-*Eco* RI fragment from JR083 was transferred to that position to give the two marker genes tandemly arranged on the same plasmid. This construct is shown in Fig. 1.

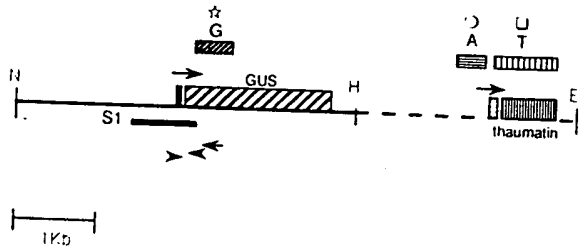

Fig. 1. Diagram of JR214. The ca. 6.7 kb insert, comprised of the JR133 construct (between *Nhe* I (N) and *Hin*d III (H) sites), and the JR083 construct (between *Hin*d III and *Eco* RI (E) sites), was placed in the polylinker of pBS + (Stratagene) as described in Materials and methods. The solid horizontal line in JR133 represents 5′ and 3′ sequences from the high-pI-barley α-amylase gene, Amy6-4, while the dashed horizontal line in JR083 represents 5′ and 3′ sequences from the low-pI- α-amylase gene, Amy32b. The JR133 solid box represents α-amylase coding sequence fused in frame to the GUS sequence (cross-hatched box). The JR083 double cross-hatched box represents PAPI coding sequences fused in frame to the coding sequence of thaumatin (box with vertical lines). The position of transcript initiation for each of the α-amylase promoters is indicated by the horizontal arrows above the two constructs. Also above the constructs are three boxes that represent the position of probes used for hybridization: 'G', specific for GUS; 'A', specific for the Amy32b promoter/upstream sequence; 'T' specific for thaumatin. Symbols above those letters (star, circle, and square, respectively) correspond to those used in Fig. 2. The thick horizontal line below the construct, labeled 'S1', represents the position of the end-labeled *Bcl* I fragment used in S1 nuclease protection assays to detect transcripts from the GUS gene (see Fig. 5). The arrow below the line indicates the position of the reverse transcriptase primer, and the two arrowheads represent the position of PCR primers used in Fig. 6.

Injection of barley tillers

Seeds from *H. vulgare* L. cv. Himalaya, 1985 crop, were obtained from the Agronomy Club, Washington State University, Pullman, WA. Plants were kept in the Biology Department Plant Growth Facility, either in a greenhouse or in a growth chamber; the temperature was maintained at 10 °C overnight, 18 °C for the day, with a 14 h photoperiod. Tillers were injected at a developmental stage when they had 5 leaves and when the developing inflorescence could be palpated between the third and second leaves (from the top); this corresponded to a time 1–2 weeks before anthesis. In collaboration with Dr Preben Bach Holm at the Carlsberg Laboratories, Copenhagen, the development of anthers from 5 floral nodes removed at this stage was determined cytologically. Anthers in each spike were essentially synchronous in their development. The microspores in every instance were at a stage corresponding to a time preceding or at the first post-meiotic mitosis (P.B. Holm, S.W. Rogers, and J.C. Rogers, unpublished data). DNA entering microspores at this stage therefore would be transmitted to both endosperm and embryo, but we do not have any information regarding the actual cell type that was transformed. A portion of 300 μl of plasmid DNA purified by CsCl banding and exclusion chromatography through an Agarose A50M (BioRad) column, 100 μg/ml in 0.1 mM EDTA pH 7.4, was injected with a standard tuberculin syringe into the hollow space in the stem immediately above the inflorescence. Care was taken not to contact the inflorescence with the needle; otherwise the inflorescence died. Grains developing from injected tillers were harvested when they were completely dry (about 5–6 weeks after anthesis). These were catalogued by placing each grain in an individual well of a 96-well microtiter dish.

Screening method to detect GUS activity

The tip of each grain opposite the embryo (about 1/4 the volume of the grain) was cut off and placed in an identical position in a duplicate microtiter dish. The remainder of each grain was returned to its catalogued well and saved for use if necessary for germination and growth of a plant. Grain ends were sterilized by washing for 1 min in 70% ethanol, then for 20 min in 0.2% $AgNO_3$, then were allowed to dry in a sterile tissue culture hood. To each well was then added 100 μl of incubation buffer: 20 mM sodium succinate pH 5.2, 10 mM $CaCl_2$, 10^{-6} M GA_3, 50 μg/ml carbenicillin, and 125 ng/ml amphotericin B. Plates were incubated at room temperature in a humidified atmosphere for 2 days; under these conditions the aleurone layers vigorously secreted α-amylase. The incubation medium was then removed, and 25 μl of homogenization buffer (50 mM sodium phosphate pH 8, 10 mM EDTA, 0.1% NP40, 0.1% sarkosyl, 1 mM phenylmethylsulfonyl fluoride (PMSF) containing 1 mM 4-methylumbelliferyl β-D-glucuronide (MUG; Sigma Chemical Co, St. Louis, MO) was added to each well. Each seed end was homogenized in its well with a teflon pestle until the aleurone was thoroughly broken. One hundred μl more of the same buffer was added to each well, and the samples were incubated overnight at room temperature. The conversion of MUG to its blue fluorescent product was assessed by removing 50 μl from each well and transferring each to identically positioned wells in a fresh microtiter dish. The contents of each well were then alkalinized by adding 5 μl of 2 M NaOH, mixed gently, and viewed on a long (>300 nm) wavelength UV light. Samples from negative controls showed no fluorescence; putative positive transformants were identified by visualizing blue fluorescence in the assay sample. The corresponding half grain containing the undisturbed embryo was then planted for subsequent Southern blot analysis. In previous work (summarized in the Introduction) where the JR133 construct was used for injection, the frequency with which a positive Southern blot was obtained from seeds screened as positive was similar to that shown below for construct 214 (data not presented). This suggested that our screen was likely to identify most seeds that carried the reporter DNA. To test this prospectively, 96 seeds from tillers injected with construct JR133 were planted without being first screened with the seed end GUS assay. Southern blots were done on DNA obtained from the 61 plants that germinated; one was positive. We therefore concluded that plants carrying the foreign DNA were present in an unselected population at a frequency (ca. 1%) that was not different from that determined with the screening plus Southern blot analysis strategy, and that the screening procedure (the seed end GUS assay) greatly increased the likelihood that a positive Southern blot would be obtained. For those reasons we believe that the screening strategy described here is a valid and unbiased approach to detect most of the plants that would carry the foreign DNA constructs described below.

Results

Experimental approach

We previously had used two different marker genes, those for thaumatin and for GUS; since in those experiments, as individual constructs, they seemed to have different stabilities in barley (data not shown), we placed them in tandem on the same plasmid for the experiments described here so that they could be compared directly. A schematic diagram of the final construct carried in pUC19 (designated JR214) is shown in Fig. 1. For each experiment, Southern blots of DNA from transformed plants were probed consecutively with three different probes specific for three different parts of the construct; these are indicated by 'G' (590 bp at the 5' end of the GUS-coding sequence), 'A' (540 bp of the Amy32b promoter and 5' mRNA untranslated sequence), and 'T' (640 bp of thaumatin sequence). The 'A' probe was used because it is a single copy sequence in the barley genome [18] and allowed us to compare the intensity of hybridization of the foreign DNA to a single-copy internal control. Although

the restriction fragments used to generate each of these probes were gel-purified, each of the probes appeared to cross-hybridize to some extent to the pUC19 vector sequences as assessed with internal control lanes containing control barley DNA spiked with added plasmid DNA. This is demonstrated, for example, in Figs. 3B and 3C (lanes marked C + P) where the 'T' and 'A' probes respectively appear to hybridize strongly to the expected 2.5 kb fragment as well as to the 7.2 kb fragment carrying the GUS and plasmid sequences. Nevertheless, it is clear that these probes provide a clear separation between fragments in barley DNA that carry 'G' sequences and those that carry the 'T' or 'A' sequences. This is clearly shown for the 'T' probe in Fig. 3A as compared to Fig. 3B, and for the 'A' probe in Fig. 3C as compared to Fig. 3D. In each instance, the 'G' probe hybridized to a 7.2 kb fragment (Fig. 3A, lane 1H11, and Fig. 3D, lane 1H11-1), while the 'T' probe (Fig. 3B, lane 1H11) and the 'A' probe (Fig. 3C, lane 1H11-1) did not. Thus, while we cannot formally exclude that any one probe might cross-hybridize to some extent to accompanying plasmid sequences present in the barley DNA, the specificity of hybridization demonstrated here allows us to make reasonable assessments as to the presence or absence of one or the other sequences.

In attempting to explain the difference in stability previously observed between the two marker genes, we noted that the very unstable GUS marker maintained the methylation phenotype characteristic of plasmid DNA, while the thaumatin marker showed some loss of N-6 methyladenine and apparent acquisition of some deoxycytidine methylation at CG dinucleotides, as judged by digestion with methylation-sensitive restriction enzymes (data not shown). For that reason, we designed the present experiments to test, in a prospective manner, whether the state of methylation of plasmid DNA used for injection influenced either the transformation frequency or the stability of the foreign DNA once it was present in the plant. We conducted a series of consecutive experiments where plasmid DNA with one modification was injected into barley tillers, and seeds developing from self-pollination on those tillers were collected and screened. At that time, new plants were injected with the next construct while the first seeds were being analyzed. This ensured that only one plasmid DNA was being used for injection at any given time. As plants germinated from grains selected by the fluorescent GUS activity screening test, the primary tiller was left undisturbed but secondary tillers were removed in the order they appeared when they contained ca. 1 g tissue and used for DNA analysis. The results of Southern blot analysis for each experiment are presented in graphic form in Fig. 2, with specific numerical analyses in Table 1. Specific examples of Southern blot results are shown in subsequent figures.

Effects of methylation of A and C residues

Table 1A summarizes the methylation patterns for the four variations tested. Construct 214 represents the plasmid as purified from a standard laboratory strain of *E. coli*, where the DNA had N-6 methyladenine in each GATC sequence (*dam* methylation) and 5-methyldeoxycytidine at the internal C of the sequence CCA/TGG (*dcm* methylation). Plasmid 214A was isolated from an *E. coli* strain deficient in *dam* and *dcm* methylation; 214AM represents this plasmid methylated *in vitro* on the internal C of the sequence CCGG with *Hpa* II methylase, while 214A-dam represents the plasmid methylated *in vitro* with *Dam* methylase. Figure 2 presents the Southern blot hybridization results so that both the frequency of transformation, as well as the stability of the foreign DNA within the plant can be visualized for each variant. The presence of a symbol means that a blot was probed with that particular probe: square = thaumatin ('T', Fig. 1), star = GUS ('G', Fig. 1), circle = Amy32b promoter ('A', Fig. 1). Open symbols mean that the results were negative while closed symbols represent a positive result.

214: dam *and* dcm *methylation.* It can be seen that 5 of the 9 plants studied for 214 transformation gave a positive signal with at least one probe

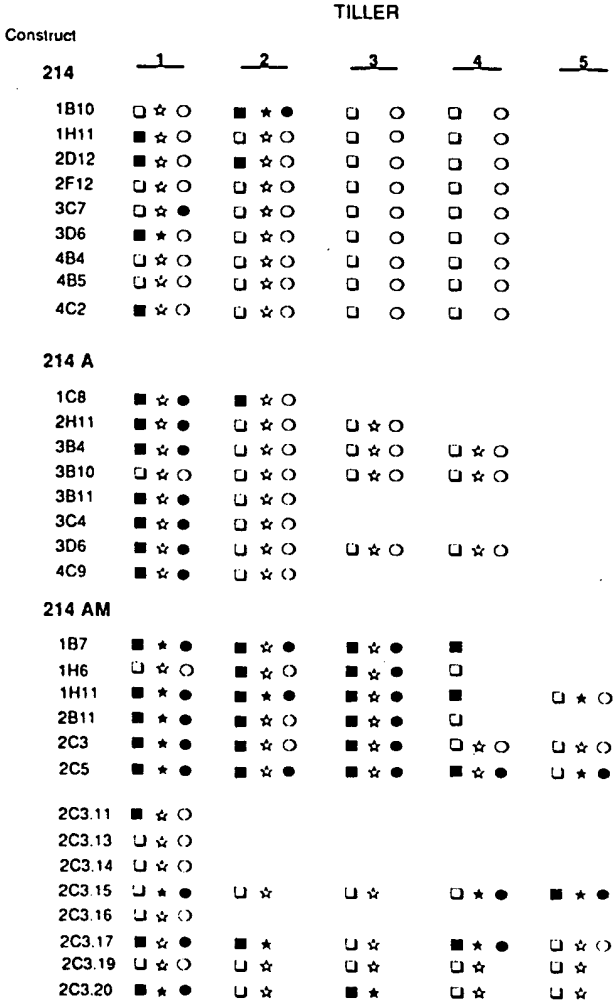

Fig. 2. Summary of Southern hybridization results for plants transformed with plasmids carrying different methylation patterns. The methylation phenotypes of constructs 214, 214A, and 214AM are detailed in Table 1. The number/letter codes under each construct number refer to putative positives from the aleurone tissue GUS assay whose embryos were germinated to give plants for Southern analysis; under 214AM, the second set of numbers (beginning 2C3.11) represent 8 progeny plants that germinated from 10 grains from the primary tiller of the 214AM transformant, 2C3. 'Tiller samples' refers to tillers removed sequentially from a plant for DNA preparation; note that the primary tiller of each plant was not disturbed, so that No. 1 refers to the first tiller that appeared after the primary tiller. Symbols under 'Tiller samples' designate results obtained from Southern blot hybridization with the 'T' probe (squares), 'G' probe (stars), and 'A' probe (circles). The presence of a symbol means that hybridization with that probe was performed: a solid symbol indicates a positive result while an open symbol means that no specific hybridizing band was detected. Usually a single Southern blot was hybridized sequentially with each of the three probes; after autoradiography, the blot was stripped prior to hybridization with a subsequent probe.

Table 1. Methylation patterns.

A. Methylation status of constructs[1]

Construct	*dam* methylation	*dcm* methylation	*Hpa* II methylation
214	+	+	0
214A	0	0	0
214AM	0	0	+
214A-dam	+	0	0

B. Comparison of transformation frequencies[2]

	214	*214A*	*214AM*	*214A-dam*
214	5/436	$p = 0.39$	**$p = 0.037$**	$p = 0.33$
214A	7/350		$p = 0.23$	$p = 0.068$
214AM	6/150			**$p = 0.012$**
214A-dam	0/166			

[1] Plasmids with different methylation patterns were prepared as follows: 214 was prepared from *E. coli* strain DH5a (Bethesda Research Laboratories). 214A was prepared from strain GM2929 (*dam*-13::Tn9 *dcm*-6 *hsd*R2 *recF*143 McrA-McrB-) [21], generously provided by Dr Martin Marinus of the University of Massachusetts, Worcester, MA. The absence of *dam* and *dcm* methylation was confirmed by digesting with *Mbo* I and *Eco* RII, respectively. 214A was methylated with *Hpa* II methylase, while 214A-dam was methylated with Dam methylase (both enzymes from New England Biolabs); completeness of methylation was documented by the absence of detectable digestion with *Hpa* II and *Mbo* I respectively.

[2] The table presents, on the diagonal, the total number of transformants as assessed by Southern analysis/total number of grains screened for each construct; if DNA samples from the first two tillers tested did not show hybridization with one of the probes, the plant was scored as negative. To the right of the diagonal are p values that statistically compare results with two different constructs. Comparison was made using Fischer's Exact Test from the SAS software package (release 6.06) carried in the Washington University Medical School VAX computer system. Results are for two-tailed analyses, except in comparisons with 214A-dam where comparisons were one-tailed. Comparisons where $p < 0.05$ are in bold type underlined; one comparison approaching this level of significance is in plain type underlined.

in DNA samples from one of the two first tillers studied; in three, only the thaumatin-specific probe hybridized. In growth subsequently tested (tiller samples 3 and 4), no detectable hybridization was obtained with any of the three probes. These results suggest that the foreign DNA persisted for a limited time in actively dividing meristematic tissue, but that it was progressively lost as this population of cells expanded. Thus tillers that grew out later were found not to have the sequences. This hypothesis is supported by data (below) demonstrating that the copy number of the foreign DNA sequences can change, as well as by expression data (also below) that argue strongly the foreign DNA is within barley itself.

214A: no methylated nucleotides. The 214A plasmid DNA, which lacked all methylation, gave a transformation frequency that was not different from that obtained with 214 ($p = 0.39$); interestingly, none of the positive DNA samples hybridized with the GUS probe, and, as with 214, subsequent growth on the plants was found to lack hybridizing sequences. Since subsequent variations used this same 214A DNA that was methylated *in vitro* to introduce methyl groups at specific sites, these results provide a basis upon which the effect of such modifications can be assessed.

214AM: Hpa *II methylation.* When 214A DNA was first methylated *in vitro* with *Hpa* II methylase and then used for transformation, a significant increase in transformation frequency was observed when compared to the results obtained with 214 ($p = 0.037$, Table 2B), but was not different from the results with 214A ($p = 0.23$). Important differences were observed with respect to the stability of the foreign DNA sequences, however. As can be seen in Fig. 2, all of the 6 plants tested by Southern hybridization were positive when the first two tillers were analyzed, and, in contrast to results with 214 and 214A, hybridizing sequences persisted in most of the tillers that were subsequently assayed.

Figure 3 presents Southern blot hybridization results from which data in Fig. 2 were derived. In each case the DNA was digested with a combi-

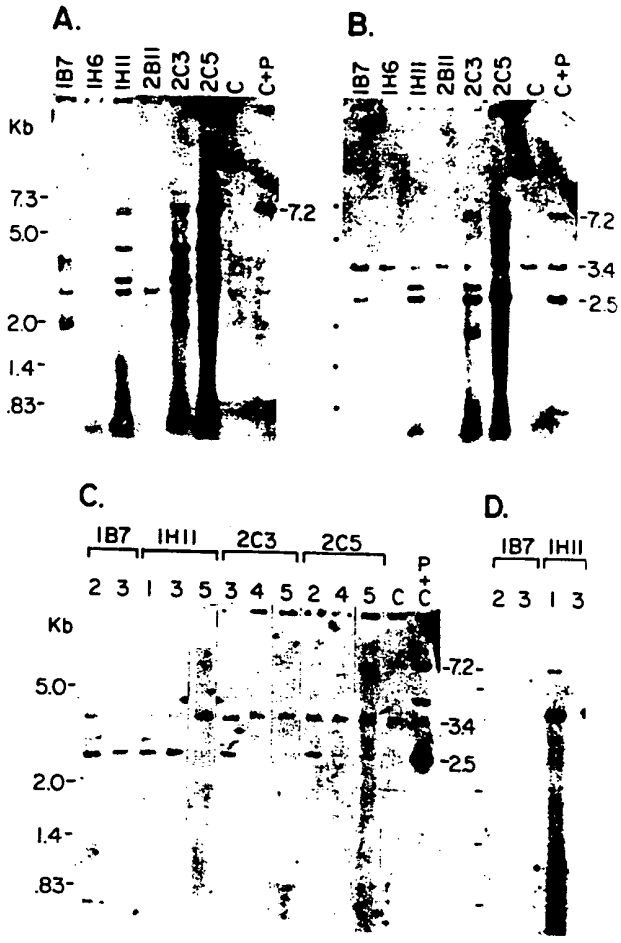

Fig. 3. Hybridization to DNA samples from plants transformed with 214AM. Numbers to the left of the panels represent the positions of *Hind* III + *Eco* RI digested fragments of bacteriophage λ DNA visualized on the ethidium bromide-stained agarose gel prior to transfer; numbers to the right represent the position of the plasmid + 'G'-hybridizing sequences (7.2 kb) or the 'A' + 'T'-hybridizing sequences (2.5 kb), as well as the position of the single copy *Hind* III genomic fragment carrying the Amy32b promoter sequence that hybridizes to 'A'. Genomic DNAs were digested with *Hind* III + *Eco* RI. Hybridization probes were: panels A and D, 'G' probe; panels B and C, 'A' probe. Panels A and B show DNA from tiller sample 1 of the 6 plants transformed with 214AM, while in panels C and D the numbers above each lane refer to the tiller samples of the transformants indicated above the brackets. Lanes labeled C contain DNA from control barley, while C + P indicates control barley spiked with about 5 copies of 214 plasmid DNA. The arrowhead in 2C5-5 indicates the position of a new, extra hybridizing band.

nation of *Hin*d III and *Eco* RI; in the original plasmid, these enzymes release a 2.5 kb fragment containing the thaumatin marker gene construct and a 7.2 kb fragment containing the GUS gene construct and the plasmid sequences (Fig. 1). Each blot carries a lane with untransformed control barley DNA (C), and a lane in which this control DNA was spiked with ca. 5 copies of the 214 plasmid DNA (C + P). Fig. 3A and 3B present DNA samples from the first tiller assayed from each of the 6 plants hybridized with the GUS ('G') probe (panel A), and with the Amy32b promoter ('A') probe (panel B). On the right of each panel is the size of the plasmid marker bands that hybridized to each probe: 7.2 kb for 'G' and 2.5 kb for 'A', with some hybridization of contaminating plasmid sequences to 7.2 kb. In addition, the position of the 3.4 kb *Hin*d III fragment in barley DNA carrying the 'A' sequence is indicated to serve as an internal control. It can be seen that DNAs from 1B7, 1H11, 2C3 and 2C5 had multiple bands that hybridized to 'G'; in general most bands also appeared to hybridize to 'A', with the prominent exception of an ~4.5 kb band in 1H11, 2C3 and 2C5. When the intensity of 'A' probe hybridization to the 3.4 kb amylase gene internal standard was compared to that of the additional bands, 1H11, 2C3 and 2C5 appeared to carry multiple copies of the foreign DNA. For both probes, the control DNA lacked the extra hybridizing bands. Results for hybridizations to samples from subsequent tillers removed from these plants are presented in Fig. 3C and 3D: panel C, 'A' probe, panel D, 'G'. Numbers above the lanes indicate the tiller samples analyzed; these correspond to the numbers in Fig. 2. It can be seen that 1B7-2&3, 1H11-3, 2C3-3, and 2C5-2, 4&5 carried 2.5 kb bands that hybridize to 'A' while 1H11-5 and 2C3-4&5 lacked any extra bands. In addition, hybridization to the 2C5-4&5 2.5 kb fragments was substantially diminished when compared to the intensity of hybridization to the 3.4 kb internal standard band, while 2C5-5 had an additional hybridizing band slightly larger than the 3.4 kb standard (asterisk). When this same blot was rehybridized with the 'G' probe (panel D), it can be seen that 1B7-2&3, and 1H11-3 (which all had bands that hybridized to 'A') lacked any 'G'-hybridizing bands; the presence of the expected 'G'-hybridizing sequences in the first 1H11 DNA sample serve as a positive control. The loss of some hybridizing bands, the diminution in intensity of hybridization of some bands, and the appearance of new hybridizing bands in different tissue samples from the same plant all indicated ongoing rearrangements of the foreign DNA.

214AM: second generation. The persistence of 214AM DNA in multiple tillers of transformed plants suggested that it might also persist into progeny of those plants. Accordingly, 10 grains from the primary tiller on 2C3 were planted; individual tillers of the 8 that germinated were studied as before; the results are in Fig. 2 for 2C3.11, and 13–20. It can be seen that four, 2C3.13, 14, 16, and 19 lacked hybridizing sequences in tissue samples tested, while 2C3.15, 17, and 20 maintained hybridization to some of the probes in some samples. Southern blot analysis of 9 DNA samples from these plants is presented in Fig. 4A and 4B; the thaumatin probe ('T', Fig. 1) was used in A, and the 'G' probe was used in B. The left lane (labeled 2C3) contains DNA from the first tiller

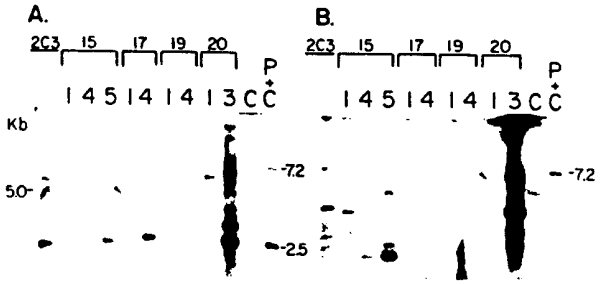

Fig. 4. Hybridization to DNA samples from progeny plants of 214AM transformant 2C3. Restriction enzyme digests, markers, and controls were as for Fig. 3. Panel A represents hybridization with the 'T' probe, while panel B shows results with the 'G' probe. The lane marked 2C3 contains DNA from tiller sample 1 of the parent plant, while the numbers above the brackets refer to individual progeny plants (2C3.15, etc). Numbers above each lane refer to tiller samples obtained from those plants. Arrowheads indicate the position of faintly hybridizing bands.

sample from the parent plant, while 15, 17, 19, and 20 represent samples from those 2C3 progeny respectively. The general conclusion to be made from these results is that the size and intensity of hybridizing bands from one tiller in one plant rarely, if at all, were the same as those from a different tiller from the same plant. Notable examples of apparently substantial increases in copy number can be seen from the 'G' hybridization pattern in 15-5 and 20-3; in the latter the 'T' probe gives similar results. In addition, continued generation of discrepancies between hybridization results with the two probes are seen; for example, 'T' hybridization was absent in 15-1 and 4, while it was clearly positive for the 'G' probe in those samples. We conclude that continued rearrangement and deletion of parts of the foreign DNA is present in these progeny plants, and that the pattern seen for at least 20-3 must represent over-replication of portions of the foreign DNA sequences.

214A-dam: dam *methylation only.* When 166 grains from tillers injected with this construct were screened with the seed end fluorescent GUS assay, no signals above baseline were observed. Three embryos from grains that gave very questionably positive signals were planted; two tillers from each of these three plants were negative by Southern blot analysis. While the statistical comparison of this result to that obtained with the 214A construct did not quite reach the 0.05 level ($p = 0.68$, Table 1B), it strongly suggests that the presence of N-6-methyladenine residues had a major negative effect on transformation efficiency and/or stability of the DNA in barley cells. The results obtained with 214A-dam were significantly different from those obtained with 214AM ($p = 0.012$, Table 1B), a result consistent with the observed significant difference between 214AM and 214 (Table 1B). Taken together, these data demonstrate that plasmid DNA methylated only on C residues at some CG dinucleotides is much more stable in barley cells than is plasmid DNA carrying some methylated adenine residues, regardless of whether that plasmid also has *dcm* methylation of C residues.

Expression of the GUS gene

We hypothesize that we were not able to demonstrate Mendelian segregation of the foreign DNA in progeny because the transforming DNA sequences were so unstable. This result might argue that the foreign DNA was not in barley but, rather, was carried in some previously undefined contaminating microorganism. We sought to analyze expression of the GUS gene, reasoning that proper initiation of transcription on the barley α-amylase promoter, and preferred expression of the foreign gene in aleurone tissue (similar to the regulation of this promoter in barley), would strongly support the concept that this unstable DNA was in barley itself. We concentrated on analysis of the GUS gene primarily because the thaumatin sequence did not have convenient restriction sites for S1 nuclease experiments and because the (G + C)-rich nature of that sequence made reverse-transcriptase-PCR analysis difficult.

Nuclease protection with end-labeled probes. In the first approach, we performed S1 nuclease-protection assays on total RNA isolated from aleurone layers treated with gibberellic acid (GA_3) as previously described [18]. Results are presented in Fig. 5; at the top is a diagram of the probe: a 5′ end-labeled 671 bp *Bcl* I fragment isolated from 214A. The cross-hatched box represents GUS coding sequences, while the solid box represents the α-amylase coding sequence fused in frame to GUS. The arrow represents the transcription initiation site [18]. This probe was denatured and hybridized either to 50 μg of RNA from control aleurone layers (C) or from plant 2C3 transformed with 214AM. The denatured S1 nuclease-resistant products were electrophoresed on a urea-acrylamide gel and identified by autoradiography. 'M' represents end-labeled *Msp* I fragments from pUC19; their sizes are indicated with open arrowheads to the right. 'P' represents undigested probe; an empty lane is between C and 2C3, and between 2C3 and P. It can be seen that only 2C3 has a protected fragment (dark arrowhead) indistinguishable in size from the 248

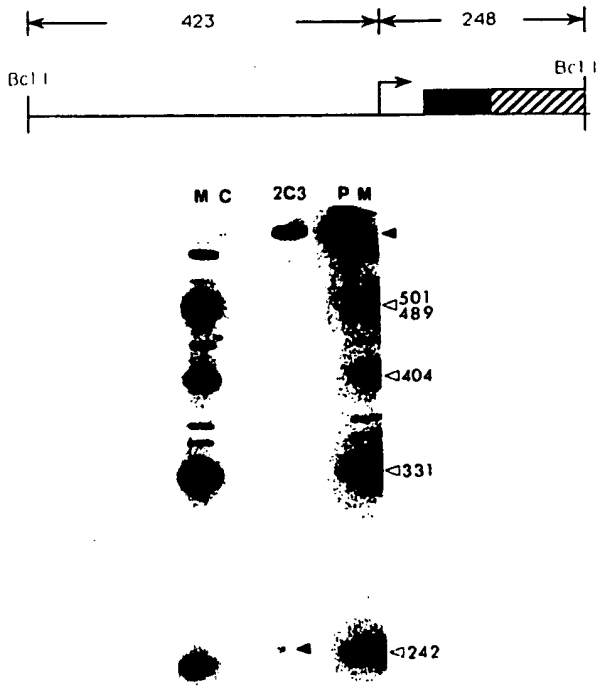

Fig. 5. S1 nuclease-protection assay to identify properly initiated GUS transcripts. A 671 bp *Bcl* I fragment was isolated from 214A, treated with bacterial alkaline phosphatase, and end-labeled with γ[^{32}P]ATP as described [18]. 100 000 cpm of gel purified, denatured probe was hybridized at 42 °C for 8 h to 50 μg of total RNA isolated from aleurone layers incubated for 24 h in the presence of 10^{-6} M GA_3; aleurone layers were from control or from 100 grains randomly selected from those produced by 5 tillers from 214AM transformant, 2C3. After digestion with 500 u/ml S1 nuclease [18], the products were analyzed on a 5% acrylamide/urea sequencing gel and identified by autoradiography for 8 days with an intensifying screen. Top: diagram of the probe fragment. The position of the 248 bp fragment that would be protected from a transcript properly initiated on the Amy6-4 promoter [18] is indicated. The cross-hatched rectangle represents GUS coding sequence, while the solid black rectangle represents α-amylase coding sequence fused in frame to GUS. Bottom: results of autoradiography. M = end-labeled *Msp* I fragments of pUC19; the size of the fragments is indicated with open arrowheads to the right. P = position of the undigested probe (solid arrowhead to right); C = control RNA, 2C3 = RNA from transformant. There are empty lanes between C and 2C3, and between 2C3 and P. The position of a ca. 248 bp protected fragment from 2C3 RNA is indicated by a solid arrowhead.

base fragment expected if the probe was protected by a transcript initiated properly on the α-amylase promoter.

Reverse transcriptase-PCR analysis. Because of the low abundance of this transcript and the relatively limited amount of RNA available from the transformed plants, we turned to an alternative strategy to analyze transcripts in other samples. The strategy is depicted in Fig. 6, top. We hybridized an oligonucleotide (identified by RT, top) complementary to the GUS sequence in the transcript to total RNA, and then extended this primer with reverse transcriptase. A portion of that reaction was then amplified by the polymerase chain reaction using a 5′ primer positioned exactly at the terminus of an authentic transcript and a 3′ primer positioned just inside the sequence used for reverse transcriptase priming; the positions of these primers are indicated by arrows that bracket the expected 322 bp PCR product. For some controls, the 5′ primer was positioned at (−204) [18] on the amylase promoter (indicated by *→). Equal amounts of the PCR products were electrophoresed in a nondenaturing acrylamide gel, transferred to nylon membrane, and hybridized with an oligonucleotide specific for the GUS sequence located immediately adjacent to the α-amylase-GUS translational fusion (indicated by 'P'). Thus, a proper 322 bp product hybridizing to this probe would be generated only if it contained three different GUS-specific sequences and one α-amylase sequence on the same transcript.

Results are presented in Fig. 6, bottom. Lanes 5 and 6 contain the products from equal amounts of 2C3-20 aleurone RNA incubated without (−rt, lane 5), or with (+rt, lane 6) reverse transcriptase. Only lane 6 has a hybridizing band of the proper size; this result demonstrates that the positive result must depend upon an RNA template, and is not caused by PCR amplification of some DNA contaminant. No hybridizing band was generated when leaf RNA (from tiller 2C3-20.5) was used (L, lane 7); similarly, no band resulted when RNA from GA-treated control aleurone layers was used (CG, lane 8). The absence of products in lanes 7 and 8 was not due to degraded RNA because a parallel S1 nuclease protection experiment to assess the abundance of the aleurain mRNA (expressed in both leaf and aleurone cells [33, 38]) demonstrated the ex-

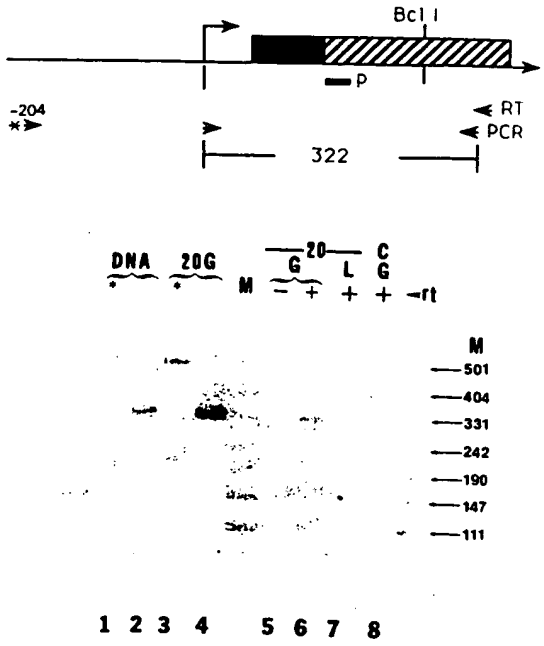

Fig. 6. Reverse transcriptase (RT)-PCR analysis of GUS transcripts expressed in aleurone layers from 2C3.20. For reverse transcriptase primer extension, 50 pmol of oligonucleotide was hybridized to 25 μg of total RNA from GA-treated aleurone or leaf tissue in 10 μl of 0.4 M NaCl, 40 mM PIPES pH 6.5, 1 mM EDTA, 80% formamide at 65 °C for 4 h, then cooled slowly over 30 min to 25 °C. After ethanol precipitation, the annealed mixture was incubated in 20 μl buffer [32] containing 50 μg/ml actinomycin D and 50 u AMV reverse transcriptase, at 37 °C for 2 h. For PCR amplification, 5 μl of the reverse transcriptase reaction mixture was diluted to 100 μl in 50 mM KCl, 10 mM Tris-Cl pH 8.3, 3 mM $MgCl_2$, containing 0.2 mM of each of the deoxynucleotide triphosphates, 50 pmol each of the oligonucleotide primers, and 3–5 u *Taq* polymerase. Amplification was for 3 cycles of 94 °C→50 °C→72 °C (for 1.5, 2.0, and 1.5 min respectively), followed by 27 cycles of 94 °C→60 °C→72 °C. Aliquots of 10 μl were then electrophoresed in a nondenaturing 5% acrylamide gel, transferred to nylon membrane (Gelman Biotrace RP) in 0.4 M NaOH, and hybridized with a kinase-labeled oligonucleotide probe in 7% SDS, 0.5 M sodium phosphate pH 7.0, 100 μg/ml salmon sperm DNA at 42 °C for 16 h. After washing twice in 2× SSC for 15 min at room temperature, the filter was washed at 55 °C for 15 min in 2× SSC; 1% SDS, then exposed for autoradiography. Top: diagram of the 5′ end of the GUS construct; symbols are as for Figs. 1 and 5. RT = primers for reverse transcriptase: 5′-TTACGAATATCTGCATCGGCGAA-3′ (nucleotides 349–317 on transcript from construct). PCR: The expected product would be 322 bp generated by a sense-strand primer (5′-ATGCATCAGTTCTCCATCGTACTC-3′ from nucleotides 1–24 of transcript) and an antisense-strand primer (5′-CTGATCGTTAAAACTGCCT-3′ from nucleotides 317–297 on transcript). In some experiments, a sense-strand primer (5′-GGCTGCAGCCATCTACATCAC-3′, marked with *) positioned at nucleotide −204 on the amylase promoter was used. The GUS-specific hybridization probe (marked P, 5′-GGGTGGTCAGTCCCTTATGT-3′) was positioned just 3′ to the amylase-GUS sequence fusion, nucleotides 138-158 on the transcript. Bottom: autoradiograph from the blot hybridization. M = end-labeled *Msp* I fragments from pUC19; sizes in bp are indicated to the right. Lanes 1 and 2: 10 pmol JR214 plasmid amplified with −204 primer (*) or 1–24 primer respectively; lanes 3 and 4: cDNA from 2C3.20 aleurone RNA amplified with same two sense-strand primers. Lanes 5–8: all used 1–24 sense-strand primer. Lane 5: 2C3.20 aleurone RNA hybridized with primer extension primer but incubated in the absence of reverse transcriptase; lane 6: incubated with reverse transcriptase. Lane 7: leaf RNA from 2C3.20 tiller sample 5. Lane 8: control aleurone RNA. The relative amount of hybridization to bands in lanes 1–4 was quantitated with a Molecular Dynamics 300A computing densitometer; the relative intensity values were: 1.0 (lane 1); 1.2 (lane 2); 1.6 (lane 3); 4.9 (lane 4).

pected amounts in each sample (data not presented). In order to assess whether properly initiated transcripts were responsible for the result in lane 6, the remainder of the reverse transcriptase products used in lane 6 were again tested by PCR: half were amplified with the upstream 5′ primer (*, lane 3) and half were amplified with the proper 5′ primer (lane 4). These products were compared to products from 100 pg of 214 plasmid DNA amplified in parallel (lanes 1 and 2). It can be seen, as expected, that approximately equal amounts of each of the expected products were amplified from the plasmid DNA. In contrast, although some larger product was generated from the 2C3-20 cDNA (lane 3), the amount of product generated with the proper 5′ primer (lane 4) was 3-fold greater as assessed by quantitation by densitometry. We conclude that some transcription was initiated from 'improper' upstream sites on the foreign gene promoter, but that most was likely to result from proper initiation. The 2C3-20 leaf RNA control excludes artifacts as a cause for the 322 bp hybridizing fragment from aleurone RNA, but does not adequately address tissue-specific expression because this RNA was made from the same tiller that showed absence of GUS-hybridizing DNA sequences (Fig. 2).

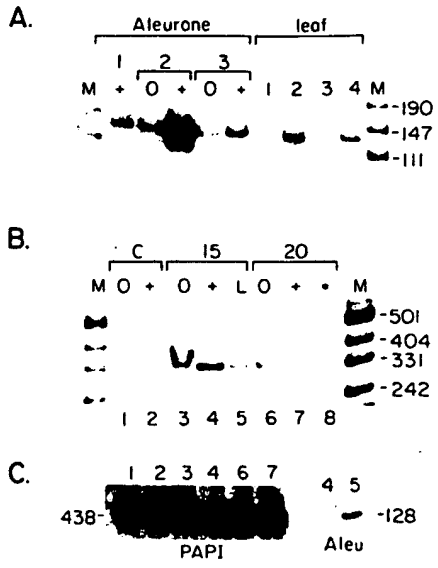

Fig. 7. RT-PCR to compare abundance of GUS transcripts in aleurone and leaf tissue from 2C3.20. Methods were the same as for Fig. 6, except that each primer extension reaction contained three different primers: one for GUS (Fig. 6), one for aleurain (nucleotides 505–489 [30], 5′-TGACGATCCCATCCTCC-3′), and one for PAPI (nucleotides 438–418 [20], 5′-AGCTTGACGGATCGTCGCTCA-3′). For PCR, equal aliquots from the cDNA reaction were used in three separate tubes to amplify the three different products. For GUS, primers and hybridization probes were as in Fig. 6. For aleurain, the sense-strand primer was 5′-GGAGGAGTTCCAGGCG-3′ (nucleotides 378–393), the antisense-strand primer was the 505–489 oligonucleotide and the hybridization probe was 5′-CCCTCCAGTCTTTGGTCT-3′ (nucleotides 490–472). For PAPI, the sense-strand primer was 5′-GACCTCCACGAGTTGCTCATCAC-3′ (nucleotides 1–23), the antisense-strand primer was the 438–418 oligonucleotide, and the hybridization probe was 5′-TTCTACTTGCTGTCAACCTGGCCG-3′ (nucleotides 176–153). Panel A: quantitation of aleurain mRNA levels in different tissues. Three different aleurone RNA preparations and 4 different leaf RNA preparations were used; for aleurone preparations 2 and 3, RNA was from layers incubated in the absence of hormone (0) or in the presence of GA for 24 h (+) while preparation 1 was from only GA-treated layers. Panel B: quantitation of GUS transcripts in different tissues. '0' and '+' refer to RNA from aleurone layers incubated in the absence or presence of GA, respectively. For each 2C3.15 (lanes 3 and 4) and 2C3.20 (lanes 6 and 7), layers were prepared from 100 grains; each aleurone layer was divided in half so that the tissue incubated without hormone was genetically identical to that incubated with GA. The 2C3.20 RNA used here was a separate preparation from that used in Fig. 6. Lane 5 represents RNA from 2C3.15 tiller sample 5. Lane 8 used the same cDNA as in lane 7, but the PCR amplification was performed with the aleurain sense-strand primer and the GUS antisense-strand primer. Panel C: PAPI and aleurain internal standards. Left: the 438 bp PAPI product was amplified from cDNAs used in lanes 1–4, 6, and 7 in Panel B to ensure that the RNA preparations and cDNA synthesis reactions were intact. Right: the 128 bp aleurain product was amplified from cDNAs used in lanes 4 and 5 to allow a quantitative comparison of the results shown in Panel B.

Quantitative estimates of foreign gene expression in aleurone and leaf tissues. We first established that our reverse transcriptase-PCR conditions would allow reasonable comparisons of mRNA abundance in different preparations, and then we used this assay to compare the level of expression of the foreign gene in aleurone tissue from progeny plant 2C3.15, and in leaf tissue (tiller sample 5) where DNA sequences hybridizing to the 'G' probe were detected (Fig. 4B). In each experiment, the initial primer extension reaction included both a primer specific for GUS, and at least one other primer that would hybridize to an internal standard mRNA. The cDNA products of this reaction were then divided into equal portions and the foreign gene and internal standard products were amplified separately using PCR. To establish the validity of the approach, we first used primers specific for the mRNA for aleurain, a thiol protease mRNA that is GA-regulated in aleurone cells but also expressed in leaf tissue [33]; results are presented in Fig. 7A. Three separate preparations of aleurone layer RNAs (labeled 1–3) and four separate leaf RNA preparations (labeled 1–4) were used. For two of the aleurone preparations (2 and 3), RNA was prepared both from untreated layers (0) and from layers treated with GA(+); for each of these pairs, it can be seen that GA-treatment increased the abundance of aleurain approximately 5–10-fold as expected [18, 28, 33]. The abundance of aleurain mRNA in GA-treated aleurone layers is about 5 times greater than in leaf [33, 38]; although there is variation between individual samples, it can be seen in Fig. 7A that the assay measures greater amounts of aleurain mRNA in the +GA aleurone samples as a group, as compared to the group of four leaf samples. We therefore

conclude that this assay provides a reasonably quantitative estimate of mRNA abundance.

In Fig. 7B are presented results measuring the relative abundance of GUS mRNA in control aleurone layers (C, lanes 1 and 2), in aleurone layers from 2C3.15 (lanes 3 and 4) and 2C3.20 (lanes 6 and 7) and in 2C3.15 tiller sample 5 (lane 5). Each aleurone layer within a set had been divided in half; each set therefore contained two identical parts, one of which (+) was incubated in the presence of GA. It can be seen that the controls were negative, while GUS RNA was detected in both 2C3.15 and 2C3.20 aleurone preparations where its abundance was not affected by GA treatment. The specificity of this result is further illustrated in lane 8 (*); this PCR reaction used the same substrate as that in lane 7, but the 5′ primer was for aleurain while the 3′ primer was for GUS. Although GUS RNA was present in 2C3.15-5 tiller RNA, its abundance was substantially less than that in aleurone RNA from the same plant. Internal standards for these samples are presented in Fig. 7C; primers for both PAPI (an aleurone-specific mRNA whose abundance is not changed by GA-treatment [23]) and for aleurain had been included in the reverse transcriptase reaction. In the panel on the left, PCR primers were specific for PAPI; the presence of similar amounts of the 438 bp product demonstrate that the control RNA preparations were intact. In the panel on the right, PCR primers amplified the 128 bp aleurain product; it can be seen that the amount of this product from the leaf RNA (lane 5) was the same or greater than that from the GA-treated aleurone RNA (lane 4). This result ensures that the difference in abundance of GUS transcripts detected in Fig. 7B was not the result of degraded or inadequate amounts of leaf RNA.

Discussion

Our data demonstrate transformation of cells in barley plants. Although the foreign DNA was unstable as judged by substantial variation in the presence of hybridizing restriction fragments among different tissue samples from the same plant, in the sizes of those fragments, and in their abundance within different tissues of the same plant, this DNA must have been associated with a centromere for much of its existence because otherwise free plasmid DNA would not have segregated properly to daughter cells and would have been diluted out and lost through the millions of cell divisions that accounted for two generations of plants. Plants where one tiller carries foreign DNA and another does not are chimeras, but we suggest that such chimerism may be due to loss of the DNA from meristematic cells by mechanisms that could also account for the extensive rearrangements that were demonstrated (see below). The plant literature equates transformation with stable chromosomal integration of the test gene [30]. This concept is based primarily upon the fact that standard transformation strategies use selectable markers; thus, only plants where the marker gene is continuously present and functional would survive selection. In contrast, our strategy allowed us to follow DNA sequences that were of no advantage to the plant. It is well established that DNA sequences may over-replicate and recombine and shuttle between extrachromosomal and chromosomal locations in higher eukaryotic cells; these processes are characteristic of gene amplification in mammals [8], and it is very probable that similar processes are used by plant cells. There are data suggesting that extrachromosomal DNA is generated by replication from parts of the nuclear genome during differentiation of meristematic root-tip cells in *Pisum sativum* [19]. In addition, sorghum suspension culture cells transformed to hygromycin- or kanamycin-resistance have been shown, in some instances, to carry extrachromosomal plasmids derived from the introduced transforming DNA [5]. Thus our finding that transforming DNA was unstable in barley is not without precedent.

Could the foreign DNA exist in a contaminating 'endophyte' [30] and not in barley cells? There are strong arguments against this possibility. Firstly, the genes had no selectable advantage for any organism; thus there is no apparent reason for a lower eukaryote or prokaryote to have in-

corporated and maintained them. Secondly, the amylase promoter-GUS reporter construct was transcribed properly in purified aleurone tissue from the barley promoter (as assessed both by S1 nuclease protection assays and by reverse transcriptase-PCR assays), and gave higher steady-state mRNA levels in aleurone tissue as compared to green tissue. Thus, an 'endophyte' would have to be able to use a barley promoter correctly and would have to be more abundant in aleurone layers than in leaf tissue. This possibility seems very remote.

Our data demonstrate that the state of methylation of plasmid DNA used in transformation experiments had a major effect on, and perhaps even determined, the transformation efficiency and/or the stability of the DNA within barley cells. We found that the methylation pattern of the marker genes in the primary transformants, as judged by Southern blot analysis of genomic DNA digested with different methylation-sensitive restriction enzymes, corresponded to that found in the plasmid DNA used for transformation (data not presented). Specifically, the presence of N-6-methyladenine within the sequence GATC was strongly associated with a low transformation efficiency and with instability of the construct within plant cells, while the absence of N-6 methyladenine and the presence of some deoxycytidine methylation (m^5C) at CG dinucleotides was associated with a high transformation efficiency (ca. 4%) and much greater stability of the DNA, such that it was transmitted to the second generation and was transcriptionally active in aleurone layers of those plants. Although this is the first direct suggestion that DNA methylation in higher plants may play an important role in protecting parts of the plant genome from deletion, a number of previous observations in the literature are consistent with this hypothesis.

First, it is important to note that higher plants have a very high degree of methylation of deoxycytidine residues, ~30%, distributed between CpG and CpXpG sequences [15]. As in vertebrates, m^5C is distributed in a non-uniform pattern, with active genes being localized in relatively hypomethylated 'CpG islands' [1]. At least in maize, however, deoxycytosine methylation does not appear to regulate tissue-specificity of expression, since genomic sequencing of a maize alcohol dehydrogenase gene in leaf tissue, where it is completely silent, demonstrated no m^5C in 900 bp 5′ to the ATG [27]. Higher plants have an added complexity, however; N-6 methylation is present on about 5% of deoxyadenosine residues [26, 37], and a substantial part of that is present in the nuclear genome [25]. The latter authors observed that transcriptional activity of nuclear genes for photosynthetic proteins was increased in suspension culture cells when the cells were treated with inhibitors that decreased the amount of dA and dC methylation. Thus, dA or dC methylation may have effects on gene transcription in some higher plant cells.

Other authors have observed that loss of activity of transposable elements in maize correlates with an increase in m^5C within those elements [7, 35]; the state of methylation of dA residues was not determined in those studies. While these results could be explained by methylation-induced suppression of transposase gene transcription from these elements [35], it is also possible that methylation could interfere in some way with the physical structure of the transposon itself, thereby altering its ability to replicate and/or to be excised. In this regard it may be important that methylation of dA and dC residues has significant effects on supercoiled plasmid sequences; the rate of cruciform extrusion was enhanced 4-fold by dA methylation, while dC methylation decreased the rate by about 2-fold [24]. These results suggest that both dA and dC methylation could substantially alter the topography of a supercoiled domain in plant cell chromatin; such effects might well influence the efficiency with which recombination or interaction with proteins such as transposases could occur. There are good examples showing that the presence of m^5C may have both negative and positive effects upon DNA/protein interactions. In mammalian cells, m^5C apparently interferes with protein binding at AP-1 and octamer binding sites [36], while other studies show that there are proteins that specifically interact with m^5C in mammalian cell nuclei [2, 20].

In addition, studies in the protozoan *Tetrahymena thermophila* demonstrate that N-6 methyladenine is used to mark specific DNA sequences within a compartment. In this organism, DNA within the germ line micronucleus is unmethylated, while DNA within the somatic macronucleus contains N-6 methyladenine; the latter pattern is established de novo when a new macronucleus develops following conjugation [4, 16]. Interestingly, a highly specific subset of rDNA genes is methylated. A monomeric form found in the newly developed macronucleus that persists for several generations before being lost is not methylated, while the genes present on the stable palindromic dimers are methylated [4]. Our data provide an experimental basis, and these examples provide a precedent upon which we base the hypothesis that methylation patterns affect the stability of foreign DNA in barley. The hypothesis requires that DNA having a methylation pattern different from that of barley genomic is 'recognized' and gradually lost from the dividing pool of meristematic cells from which new differentiated tissue develops. It is consistent with the results of Antonelli and Stadler [3] who showed that maize genomic DNA carrying an antibiotic resistance gene is orders of magnitude more efficient than plasmid DNA isolated from *E. coli* in transforming maize suspension culture cells.

The high frequency with which we obtained transformed grains from injected tillers, coupled with the observations of others that *Agrobacterium* can readily transfer DNA into maize and other cereals [9, 12], indicate that it is relatively easy to introduce foreign DNA into the cells of cereal plants. Under these circumstances it is reasonable to hypothesize that the plants would have evolved a system to protect their genomes from deleterious mutations induced by frequent insertions of foreign DNA. Monocotyledonous plants, especially the cereals, have an unusual genome organization where structural gene sequences (except for those for endosperm storage proteins) are very (G + C)-rich [22, 23, 38]. (This feature is shared by the thaumatin coding sequence but not the GUS sequence.) A detailed analysis of dC methylation within these genes has not been reported but logically would accompany a concentration of dC residues; our results with methylation-sensitive enzymes in other work (data not shown) indicate that at least some are methylated. DNA from an invading microorganism would not be likely to share either this pattern of (G + C) content or methylation typical of higher plants. Perhaps this would explain why the thaumatin sequence appeared to be more stable than the GUS sequence in our experiments. These differences may provide clues that will help to identify the specific mechanisms responsible for instability of foreign DNA in our system.

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